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Highly Sensitive Measurement of Glomerular Permeability in Mice with Fluorescein Isothiocyanate-polysucrose 70

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Corresponding Author:	Lorenz Sellin, M.D. Heinrich-Heine-Universitat Dusseldorf Duesseldorf, NRW GERMANY
Corresponding Author's Institution:	Heinrich-Heine-Universitat Dusseldorf
Corresponding Author E-Mail:	Lorenz.Sellin@med.uni-duesseldorf.de
Order of Authors:	Eva Koenigshausen
	Sebastian A. Potthoff
	Magdalena Woznowski
	Johannes Stegbauer
	Lars C. Rump
	Lorenz Sellin, M.D.
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Cover Letter

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Klinik für Nephrologie

Direktor der Klinik
Univ.-Prof. Dr. L. C. Rump

European Hypertension Excellence Centre

Sekretariat
Frau Janssen
Tel.: (0211) 81-17726
Fax: (0211) 81-17722
e-mail:
Christian.Rump@med.uni-duesseldorf.de
Liliane.janssen@med.uni-duesseldorf.de

Stellvertreterin des Klinikdirektors und leitende Oberärztin
Prof. K. Ivens (0211) 81-16146
Geschäftsführender Oberarzt
Prof. L. Sellin (0211) 81-17723
Oberärzte:
Dr. A. Hansen (0211) 81-08136
Dr. S. Kücükköylü (0211) 81-18928
Dr. S. Potthoff (0211) 81 18928
PD Dr. J. Stegbauer (0211) 81-17502
Dr. M. Woznowski (0211) 81-04861
Oberarztsekretariat (0211) 81-17773

Stationen
ZA03 (Notaufnahme)
Tel.: (0211) 81-17012
Fax: (0211) 81-19518
MD 01 (Dialyseabteilung):
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ME05:
Tel.: (0211) 81-18927
Fax: (0211) 81-19519

Ambulanzen
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Fax: (0211) 81-19282
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Tel.: (0211) 81-18923 (Hämodialyse)
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Funktionsbereiche
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Tel.: (0211) 81-18910

Wissenschaftliches Labor:
Tel.: (0211) 81-10841, -18627

Studienzentrum:
Tel.: (0211) 81-17770

Webseiten der Klinik:
www.uniklinik-duesseldorf.de/nephrologie

UKD • Klinik für Nephrologie • Postfach 101007 • 40001 Düsseldorf

To
Ron Myers, Ph.D.
Science Editor
JoVE
1 Alewife Center, Suite 200
Cambridge, MA 02140
USA

Ansprechpartner:	Durchwahl:	E-Mail:	Datum:
Prof. Dr. Lorenz Sellin	0211 81 17726	Lorenz.sellin@med.uni-duesseldorf.de	10.12.2018

Manuscript Submission: „Highly sensitive measurement of glomerular permeability in mice with FITC-ficoll 70“

Dear Dr. Myers,

Thank you for the opportunity to revise the manuscript with the title „Highly sensitive measurement of glomerular permeability in mice with FITC-ficoll 70” after its review with by JoVE.

We thoroughly followed the reviewer comments. We conduct numerous additional and novel experiments to address the reviewer’s points.

All findings are documented within the rebuttal letter and two additional figures for the reviewers.

We also added a supplemental figure to the submission. A manuscript file with marked changes has also been added.

We feel that the manuscript has improved substantially by the editorial and reviewer comments. We hope that the manuscript is now suitable for publication with JoVE.

If there are any further questions please don’t hesitate to contact me.

Yours Sincerely,

Lorenz Sellin, MD

Authors contributions:

Study conception and design: L.S., E.K.

Acquisition of data: S.A.P., E.K.

Analysis and interpretation of data: E.K., L.S., J.S.,

Drafting of manuscript: E.K.

Critical revision: L.C.R., L.S., J.S., M.W., S.A.P.

TITLE:

Highly Sensitive Measurement of Glomerular Permeability in Mice with Fluorescein Isothiocyanate-polysucrose 70

AUTHORS AND AFFILIATIONS:

Eva Königshausen[1], Sebastian A. Potthoff[1], Magdalena Woznowski[1], Johannes Stegbauer[1], Lars C. Rump[1], Lorenz Sellin[1]

[1]Department of Nephrology, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany

Corresponding author:

Lorenz Sellin (lorenz.sellin@med.uni-duesseldorf.de)

E-mail addresses of co-authors:

Eva Königshausen (eva.koenigshausen@med.uni-duesseldorf.de)
Sebastian A. Potthoff (sebastian.potthoff@med.uni-duesseldorf.de)
Magdalena Woznowski (magdalena.woznowski@med.uni-duesseldorf.de)
Johannes Stegbauer (johannes.stegbauer@med.uni-duesseldorf.de)
Lars C. Rump (christian.rump@med.uni-duesseldorf.de)

KEYWORDS:

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SUMMARY:

Here, we present a protocol to test glomerular permeability in mice using a highly sensitive, nonradioactive tracer. This method allows repetitive urine analyses with small urine volumes.

ABSTRACT:

The loss of albumin in urine (albuminuria) predicts cardiovascular outcome. Under physiological conditions, small amounts of albumin are filtered by the glomerulus and reabsorbed in the tubular system up until the absorption limit is reached. Early increases in pathological albumin filtration may, thus, be missed by analyzing albuminuria. Therefore, the use of tracers to test glomerular permselectivity appears advantageous. Fluorescently labeled tracer fluorescein isothiocyanate (FITC)-polysucrose (i.e., FITC-Ficoll), can be used to study glomerular permselectivity. FITC-polysucrose molecules are freely filtered by the glomerulus but not reabsorbed in the tubular system. In mice and rats, FITC-polysucrose has been investigated in models of glomerular permeability by using technically complex procedures (i.e., radioactive measurements, high-performance liquid chromatography [HPLC], gel filtration). We have modified and facilitated a FITC-polysucrose tracer-based protocol to test early and small increases in glomerular permeability to FITC-polysucrose 70 (size of albumin) in mice. This method allows repetitive urine analyses with small urine volumes (5 µL). This protocol contains information on how the tracer FITC-polysucrose 70 is applied intravenously and urine is collected

via a simple urinary catheter. Urine is analyzed via a fluorescence plate reader and normalized to a urine concentration marker (creatinine), thereby avoiding technically complex procedures.

INTRODUCTION:

Functional or structural defects within the glomerular filtration barrier increase glomerular permeability to albumin, resulting in the detection of albumin in the urine (albuminuria). Albuminuria predicts cardiovascular outcome and is an important marker for glomerular injury[1]. Even low levels of albuminuria, lying within the normal range, are associated with an increased cardiovascular risk[1].

Under physiological conditions, albumin is filtered through the glomerulus and is almost completely reabsorbed in the tubular system[2,3]. In mice, the detection of albumin in the urine is usually performed by an albumin enzyme-linked immunosorbent assay (ELISA) from 24 h of urine collection. If urine from a 24 h urine collection or spot urine is used, small differences in albumin concentrations may be missed due to assay sensitivity problems. Most researchers, therefore, use animal models in which albuminuria is induced by robust renal injury due to toxins, drugs, and renal surgery.

Therefore, the finding of a sensitive method to detect small and transient changes in glomerular permeability is very important to the field. Rippe et al. have presented a rat model to test glomerular permeability by applying a fluorescently labeled tracer, namely FITC-polysucrose 70 (i.e., FITC-Ficoll 70), at the size of albumin[4]. The tracer application allows the testing of short-term changes in glomerular permeability (within minutes) and is very sensitive[4]. Two studies have used the tracer method in mice[5,6]. Despite its benefits, this method, unfortunately, has disadvantages: it is technically very complex, radioactive, and invasive. Further analysis of the urine is only accomplished by using gel filtration or size-exclusion HPLC[5,6].

Within this paper, we present an alternative, sensitive, nonradioactive, and fast method to measure glomerular permeability in mice using fluorescently labeled FITC-polysucrose 70. By introducing a transurethral catheter, urine collection is less invasive than bladder puncture, urethrotomy, and suprapubic catheter application, and allows urine collection at least every 30 min. Urine analysis is performed from small amounts (5 µL) using a fluorescent plate reader. Tracer concentrations in the urine are normalized to creatinine concentrations in the urine using an enzymatic creatinine assay.

Therefore, this novel method offers a sensitive tool to study early glomerular injury with increased glomerular permeability.

PROTOCOL:

The investigations were conducted according to the guidelines outlined in the Guide for Care and Use of Laboratory Animals (US National Institutes of Health Publication No. 85-23, revised 1996). All animal experiments were performed in accordance with the relevant institutional approvals (state government Landesamt für Natur, Umwelt und Verbraucherschutz [LANUV] reference number 84-02.04.2012.A397).

1. Preparation of instruments, solutions, and equipment

1.1. Reconstitute FITC-polysucrose 70 with 0.9% sterile sodium chloride (NaCl) to a final concentration of 10 mg/mL (i.e., 100 mg in 10 mL of NaCl).

1.2. Dialyze FITC-polysucrose 70 solution to remove free FITC molecules overnight at 4 °C (molecular weight cut-off [MWCO] at 10,000). Use 1 L of 0.9% sterile NaCl per 10 mL of FITC-polysucrose 70 under constant stirring. Protect from light. Aliquot the dialyzed FITC-polysucrose 70 and store it at -20 °C.

1.3. For the FITC-polysucrose 70 bolus, add 4 µL of 10 mg/mL FITC-polysucrose 70 solution to 996 µL of 0.9% NaCl (the final concentration of FITC-polysucrose 70: 40 µg/mL).

1.4. For the equilibration infusion solution, add 20 µL of a 10 mg/mL FITC-polysucrose 70 solution to 9.98 mL of 0.9% sterile NaCl yielding to a final concentration of 20 µg/mL.

1.5. For the experimental solution, add drugs or substances to the infusion solution (e.g., for angiotensin II [Ang II] [100 ng/kg/min] for a 25 g mouse, add 3 µL of Ang II of a 1 mM solution).

1.6. For the surgery, prepare one shaver, two surgical clamps, one pair of surgical scissors, two tweezers, two fine tweezers, one pair of fine scissors, and swabs. Prepare two 10 cm silk threads (4-0 to 6-0) for ligation procedures.

1.7. For the placement of a central venous catheter, prepare a 10 mL syringe with a 21 G needle. Place the tip of the needle in a 30 cm-long catheter (with an inner diameter [ID] of 0.58 mm). Connect the 0.58 mm catheter to a 10 cm catheter (with an ID of 0.28 mm). Cut the tip of the smaller catheter oblique to create a sharp tip that is introduced into the jugular vein.

1.8. Prepare the anesthesia (i.e., intraperitoneal anesthesia ketamine, 100 mg/kg of body weight, and xylazine, 5 mg/kg of body weight).

1.9. Prepare a 22 G angiocatheter by discarding the needle and marking the catheter 1 cm from the tip. Preheat the heating pad to 37 °C.

1.10. Prepare a blood pressure device and change the blood-pressure-measuring membrane if necessary.

2. Preparation phase

2.1. Urinary catheter

NOTE: Section 2.1 follows the protocol as described by Reis et al.[7]. **Figure 1** and **Supplemental Figure 1** show the placement of a urinary catheter in female mice.

2.1.1. Anesthetize the mouse with ketamine/xylazine (see step 1.8). Use the toe-pinch test to confirm proper anesthesia. To maintain anesthesia, repeat the anesthesia (e.g., with half the dosage) after 60 min.

NOTE: Female FVB mice are used in this protocol.

2.1.2. Position the mouse in dorsal recumbency on a 37 °C heating pad. Tighten the lower abdomen and find the urethral ostium (e.g., under a microscope).

2.1.3. Use the plastic part of the catheter of a 22 G angiocatheter and lubricate it with xylocaine gel. Introduce it carefully 3 mm into the urethral ostium while paralleling the distal urethral axis (**Figure 1A**).

2.1.4. Turn the top of the angiocatheter 180° by keeping the tip within the urethral ostium and maintaining the axis of the urethra (**Figure 1B**).

2.1.5. Introduce the catheter 7 mm further into the mouse so that it is placed within the bladder (**Figure 1C**). Do not force the catheter beyond resistance. Correct the position of the catheter from the beginning if resistance is felt. Take note that if the position of the urinary catheter is correct, urine might already appear within the catheter.

2.1.6. Place a 1.5 mL brown tube over the top of the angiocatheter to collect the urine. Apply 1 mL of 0.9% NaCl intraperitoneally to enhance urine production.

2.2. Central venous catheter

2.2.1. Shave the neck of the mouse and place it in recumbency with the head toward the surgeon. Hyperextend the head of the mouse with a tape.

2.2.2. Disinfect the neck with 70% isopropanol. Make a small skin incision (5 mm) below the jawline, using a tweezer and a pair of scissors. Cut the skin approximately 1 cm in the direction of the sternum until the middle of the sternum is reached.

2.2.3. Carefully dissect the skin on the left side of the neck, using a pair of scissors. Make a rectangular incision of the skin to the right side of the mouse to expose the soft tissue of the neck. Use a pair of scissors and a tweezer. Fix the skin flaps with two clamps.

NOTE: The jugular vein runs along the left side of the thyroid gland or is slightly covered by the right lobe of the thyroid gland. After this step, use a microscope for surgery.

2.2.4. Carefully expose the jugular vein by blunt preparation, using the tip of the fine tweezer. Avoid injury to vein branches.

NOTE: It might be necessary to remove tissue with fine scissors. Be careful when using scissors to remove tissue as it increases the risk of bleeding.

2.2.5. Place and close a ligature with a silk thread (4-0 to 6-0) at the distal part of the visible jugular vein (toward the head of the mouse). Put tension on the ligature by fixing the silk thread with a tape to ensure slight tension on the jugular vein. Prepare a ligature around the proximal part of the jugular vein.

2.2.6. Fill the catheter with the equilibration infusion solution (see step 1.4) and fix the catheter with a tape so that the catheter is aligning the jugular vein. Control for bubbles to avoid air embolism.

2.2.7. Lift the jugular vein with fine tweezers at the site of insertion (the insertion site is 1–2 mm proximal of the ligature). Align the tubing parallel to the jugular vein. Puncture the jugular vein, aiming for the lumen, and insert for approximately 2–4 mm, paralleling the axis of the jugular vein. Avoid any brisk movements.

2.2.8. Close the ligature to fix the catheter. Control for tightness of the ligature by carefully viewing through the microscope. Put a damp swab over the site of surgery.

2.3. Blood pressure measurement

2.3.1. Place the tail-cuff at the bottom of the mouse tail while the mouse lays in dorsal recumbency. Start the measurements and repeat them 10x per time point. Build a mean from the measurements.

2.3.2. Adjust the position of the tail-cuff if blood pressure measurements do not seem correct and false data are produced.

3. Equilibration phase

3.1. Change the urinary collection tube before the equilibration phase starts and place it on ice (urine 0 min).

3.2. Introduce a 10 mL syringe filled with FITC equilibration phase solution (see step 1.4) with a 21 G needle and the larger catheter (with an ID of 0.58 mm) and place it into the syringe pump.

3.3. Fold a swab and put it around the small-vessel catheter (with an ID of 0.28 mm) that is introduced into the jugular vein. Lay the catheter inside the swab. Place a clamp over the swab to abolish retrograde blood flow or air embolism. Connect a 27 G needle with a 1 mL syringe filled with the FITC bolus to the end of the small-vessel catheter.

3.4. Open the clamp and apply the FITC bolus (100 µL). Close the clamp again and connect the larger catheter with the smaller catheter. Start the syringe pump with 0.008 mL/min (0.480 mL/h). Continue the infusion for 60 min.

4. Experimental phase

NOTE: In this phase, the effect of drugs on glomerular permselectivity can be investigated.

4.1. Change the urinary tube (time point 0 min) to another 1.5 mL brown tube. Put a swab around the small-vessel catheter and close a clamp as indicated in step 3.3.

4.2. Disconnect the large catheter from the small-vessel catheter. Change the syringes to the experimental phase solutions (see step 1.5). Let the infusion pump run so that the large catheter is filled with the equilibration solution.

4.3. Reconnect the catheters while avoiding air embolism and reopen the clamp. Start the syringe pump at 0.008 mL/min.

4.4. Continue running the syringe pump for 60 min and collect the urine within the urinary tube thereafter (urine 60 min). Place the urine tube on ice.

4.5. Sacrifice the mouse by decapitation during anesthesia.

5. Urine analysis

5.1. Fluorescence measurement

5.1.1. Thaw the urinary pool and dilute it 1:10 with phosphate-buffered saline (PBS).

NOTE: The urinary pool is a pool of urine that has been collected from healthy mice in a 12–24 h urine collection in a metabolic cage.

5.1.2. Prepare standards for the fluorescence measurement. Take 10 brown 1.5 mL tubes and label them for blank (1:10 diluted urine pool) and the following FITC-polysucrose 70 concentrations (0.625, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 µg/mL).

5.1.3. Pipet 246 µL of diluted urine pool into a 1.5 mL brown tube and add 4 µL of the FITC-polysucrose 70 stock concentration (see step 1.1) to get a final concentration of 160 µg/mL FITC-polysucrose 70. Pipet 100 µL of the diluted urine pool into all other tubes.

5.1.4. Dilute 1:2 by pipetting 100 µL of the 160 µg/mL FITC-polysucrose 70 tube into the 80 µg/mL tube and continue with the other concentrations. Assure proper mixture of the diluted concentrations (e.g., by vortexing the tube before continuing).

5.1.5. Dilute the mouse urine samples (0 min, 60 min) 1:10 with diluted urinary pool.

5.1.6. Pipette 5 µL of each standard FITC-polysucrose 70 concentration and of the mouse urine samples as triplicates in a black 384-well plate. Centrifuge the plate at 1,000 x *g* for 15 s to avoid bubbles.

5.1.7. Analyze the 384-well plate in a plate reader. Perform excitation at 496 nm and measure the fluorescence at 525 nm.

5.2. Creatinine measurement

5.2.1. Follow the manufacturer's instructions. In brief, prepare creatinine standards by diluting 10 µL of the 100 mM creatinine standard solution with 990 µL of creatinine assay buffer to prepare a 1 mM standard solution.

5.2.2. Add 0, 2, 4, 6, 8, and 10 µL of the 1 mM creatinine standard solution into a 96-well plate to generate 0, 2, 4, 6, 8, and 10 nmol/well standards, respectively. Add creatinine assay buffer to each well to bring the volume to 50 µL.

5.2.3. Prepare the reaction mixes by adding 42–44 µL of creatinine assay buffer, 2 µL of creatinase, 2 µL of creatininase, 2 µL of creatinine enzyme mix, and 2 µL of creatinine probe. For the blank, use 44 µL of creatinine assay buffer, 2 µL of creatinase, 2 µL of creatinine enzyme mix, and 1–2 µL of creatinine probe.

5.2.4. Add 50 µL of the appropriate reaction mix to each well in the 96-well plate and incubate it for 60 min on a horizontal shaker at 37 °C. Protect the plate from light during the incubation. Measure the absorbance at 570 nm on a plate reader.

5.2.5. Calculate the creatinine concentrations by subtracting the blank value from all readings. The result will have the unit nanomoles/microliter. Multiply the concentration of creatinine by the molecular weight of creatinine (113.12 ng/nmol) to receive the unit nanograms/microliter.

6. Data analysis

6.1. Calculate the FITC-polysucrose 70 urine concentration from the standard curve.

6.2. Reference the FITC-polysucrose 70 urine concentrations to creatinine concentrations in the representative urine sample.

6.3. Reference the urine 60 min samples to urine 0 min samples of controls and treated mice.

REPRESENTATIVE RESULTS:

As depicted in **Figure 2**, the method to test glomerular permeability in mice is built up in three phases. The first phase is called the preparation phase, in which a urinary catheter and a central

venous catheter are placed. The second phase is called the equilibration phase, starting with an intravenous bolus injection of FITC-polysucrose 70 and followed by the continuous infusion of FITC-polysucrose 70 for 60 min. The last phase is called the experimental phase. In this phase, the infusion of FITC-polysucrose 70 is continued and drugs or other substances can be tested for influencing glomerular permeability. Urine is collected at the end of each phase.

To investigate glomerular permeability within this tracer model, it is essential to place a urinary catheter properly and without injuring the mucosa. The placement of a urinary catheter into a female mouse bladder is demonstrated in **Figure 1**. The catheter is placed 3 mm into the urethral ostium, parallel to the craniocaudal urethral axis (**Figure 1A**). The catheter is turned 180° toward the tail of the mouse (**Figure 1B**) and introduced 7 mm further into the bladder, parallel to the murine spine (**Figure 1C,D**).

As described above, previous methods to test glomerular permeability in mice used HPLC to purify FITC-polysucrose 70 signals from urine samples[5,6]. As this method uses fluorescence measurement without a previous purification of the tracer, PBS, mouse urine, and FITC-polysucrose 70 in mouse urine were analyzed. **Figure 3A** shows the fluorescence scan of PBS with a fluorescence peak at 325 nm, which seems to be an effect of the excitation flash at 290 nm. The fluorescence scan of native mouse urine shows a fluorescence maximum at 395 nm (**Figure 3B**). FITC-polysucrose 70 dissolved in mouse urine displays a fluorescence maximum at 525 nm (**Figure 3C,D**). **Figure 3C** shows that mouse urine does not disturb the fluorescence measurement of FITC-polysucrose 70 in mouse urine. Increasing concentrations of FITC-polysucrose 70 show an increased fluorescence intensity (**Figure 3E**).

To prove that this method is capable of detecting differences in glomerular permeability, Ang II was applied in the experimental phase of the model. Ang II increased the glomerular permeability in mice 60 min after a continuous administration in nonblood-pressure-relevant dosages (**Figure 4A,B**). The increase in glomerular permeability due to Ang II could be blocked by an Ang II-receptor blocker (ARB), namely candesartan (**Figure 4A**). Ang II washout decreased glomerular permeability (**Figure 4A**). Blood pressure was measured via the tail-cuff method and did not show significant differences between the groups (**Figure 4B**). Polysucrose 70- and FITC-polysucrose 70-infused animals serve as controls for FITC-polysucrose 70- and Ang II-treated animals (**Figure 4C**).

FIGURE LEGENDS:

Figure 1: Placement of a urinary catheter in female mice. Lateral view of the mouse abdomen. (**A**) The marked and lubricated catheter is introduced 3 mm into the external urethral ostium of a female mouse. The craniocaudal axis of the urethra is paralleled with the catheter. Careful tension on the lower abdomen with the left index finger facilitates the introduction of the catheter into the external urethral ostium. (**B**) The catheter is turned 180° toward the tail of the mouse. The tip of the catheter remains 3 mm within the external urethral ostium. (**C**) The catheter is now introduced 7 mm further into the bladder. The direction of the catheter is aligned with the murine spine. (**D**) Ventral view of the mouse abdomen. The catheter is placed 10 mm into the mouse. (**E**) Urine appears with a correct insertion into the bladder.

Figure 2: Schematic illustration of the experimental procedures on a timeline. After narcosis of the mouse, a urinary catheter is placed. The central venous catheter is implanted and baseline urine is obtained (0 min). Afterward, the FITC-polysucrose 70 bolus is applied via the central venous catheter and a continuous infusion of FITC-polysucrose 70 is started. The equilibration phase for FITC-polysucrose 70 lasts 60 min. Urine is collected after the equilibration phase (0 min) and before the experimental phase starts. Within this phase, drugs or other substances (like angiotensin II) can be applied. At the end of the experimental phase, urine is obtained for analysis (60 min).

Figure 3: Fluorescence of PBS and mouse urine with and without FITC-polysucrose 70. (**A**) Fluorescence frequency scan (excitation of 290 nm, emission of 325–700 nm) of PBS shows a signal at 325 nm, which seems to be an effect of the excitation flash at 290 nm. (**B**) In the frequency scan of mouse native urine (urine pool diluted at 1:10 and 1:20), there is a signal with a maximum at 395 nm, probably caused by urinary protein autofluorescence. (**C**) Mouse urine containing FITC-polysucrose 70 (40 µg/mL) shows a signal peak at 525 nm that does not interfere with the measurement of the urine autofluorescence. (**D**) Magnification of the FITC-polysucrose 70 fluorescence peak depending on the emission wavelength. Different concentrations of FITC-polysucrose 70 (1.25, 2.5, 5, 10, 20, and 40 µg/mL) are displayed. (**E**) Increasing FITC-polysucrose 70 concentrations show an increase of fluorescence intensity at the emission of 525 nm.

Figure 4: Angiotensin II (Ang II) increases glomerular permeability. (**A**) Ang II significantly increases the glomerular permeability in mice, measured by FITC-polysucrose 70 detection in the mice's urine (mean + SEM; $n = 5$; $p < 0.004$, tested by Kruskal–Wallis test). FITC-polysucrose 70 concentrations in the urine were referenced to urine creatinine concentrations. The white columns (0 min) represent FITC-polysucrose 70 levels before the start of Ang II stimulation, the black columns (60 min) represent FITC-polysucrose 70 levels 60 min after the start of Ang II stimulation, and the grey columns (120 min or +60 min) after an additional 60 min of Ang II stimulation or 60 min of Ang II washout. (**B**) Systolic blood pressure was monitored by the tail-cuff method (mean + SEM). No significant blood pressure differences between the control and Ang II-treated groups were noted. (**C**) Polysucrose 70 and FITC-polysucrose 70 do not alter glomerular permeability in mice significantly. Ang II increases glomerular permeability in mice significantly (mean + SEM; $n = 7$, $p < 0.005$). This figure has been modified from Konigshausen et al.[8].

Supplemental Figure 1: Schematic diagram of the placement of a urinary catheter in female mice. Lateral view of the mouse abdomen. (**A**) The marked and lubricated catheter is introduced 3 mm into the external urethral ostium of a female mouse. The craniocaudal axis of the urethra is paralleled with the catheter. Careful tension on the lower abdomen with the left index finger facilitates the introduction of the catheter into the external urethral ostium. (**B**) The catheter is turned 180° toward the tail of the mouse. The tip of the catheter remains 3 mm within the external urethral ostium. (**C**) The catheter is now introduced 7 mm further into the bladder. The direction of the catheter is aligned with the murine spine. (**D**) Ventral view of the mouse abdomen. The catheter is introduced 3 mm into the external urethral ostium of a female mouse.

(**E**) After the catheter is turned 180°, it is introduced 7 mm further into the mouse bladder. The direction of the catheter is aligned with the murine spine. This figure has been modified from Reis et al.[7].

DISCUSSION:

The presented method enables the investigator to test glomerular permeability in mice in a very sensitive manner using a tracer. With this method, short-term increases in glomerular permeability can be diagnosed using only small amounts of urine. The most critical steps for successfully mastering this technique are 1) developing manual expertise in mouse surgery, especially in the cannulation of a central vein, 2) placing the urinary catheter without harming the mucosa, and 3) manual expertise in handling 384-well plates with small volumes of samples.

When placing the central venous catheter, it is essential that the catheter does not penetrate the jugular vein. To avoid penetration, we recommend putting tension on the jugular vein with a distal ligature (see step 2.2.7) and to lift the jugular vein with fine tweezers to extend the lumen of the jugular vein. To keep the insertion site small, try to avoid gross movements while inserting the catheter and always ensure the best view of the surgical field by removing extra tissue. The most critical step in the placement of the urinary catheter is the final insertion into the bladder. If resistance is felt, we recommend starting over from the beginning in order not to cause false tracks and injury to the mucosa. Catheter rotation, lubrication, and gentle movements, as well as training, help in difficult cases[7].

FITC-polysucrose 70 is a fluorescently labeled, branched, and cross-linked polymer of sucrose and epichlorohydrin[9]. It behaves in solution as a globular molecule with a spherical shape and with a low shape asymmetry but with a molecular deformability[9]. Like other sucrose molecules, FITC-polysucrose 70 is filtered by the glomerulus and not reabsorbed in the tubular system[4]. To avoid free FITC molecules in the infusion, we dialyzed the FITC-polysucrose 70 solution before application to the mice. It is possible to store dialyzed FITC-polysucrose 70 molecules at -20 °C for months. As FITC-polysucrose 70 is applied intravenously in this and other protocols, it is essential that no blood contaminates the urine samples. Therefore, the urinary catheter needs to be placed with caution and anticoagulant substances within the experiment should be avoided. The standard curve for FITC-polysucrose 70 is dissolved in mouse urine pool to get the most accurate results. Due to concentration differences in the urine at different experimental time points and between groups, FITC-polysucrose 70 concentrations need to be referenced to a marker in the urine that reflects urine concentration (e.g., creatinine). Interference of FITC-polysucrose 70 fluorescence with the measurement of creatinine in an enzymatic assay is unlikely.

The analysis of FITC-polysucrose 70 fluorescence should be performed in black 384-well plates as small urine volumes of 5 µL can be measured in these plates. We do not recommend reusing the same wells within the 384-well plates, even after washing the plates, as fluorescence is still measurable. As bubbles interfere with the fluorescence reading, the plates should be centrifuged before analysis. Alternatively, bubbles can be destroyed manually with the tip of a pipette.

The use of polysucroses to test glomerular permselectivity was described almost 40 years ago[10]. Fluorescently labeled polysucrose has been investigated in glomerular injury studies almost exclusively in rats in the past years[4,11–20]. This may have anatomical reasons due to easier surgical procedures in rats than in mice. In rats, urethrotomy is used to obtain urine samples[4,5,9,12–14,21]. To analyze plasma and urine samples, probes are subjected to high-performance size exclusion chromatography[4,5,9,12–14,21]. In addition, the glomerular filtration rate (GFR) is measured by radioactive ^{51}Cr-ethylenediamine tetraacetic acid (EDTA)[4,5,9,12–14,21]. Two previous studies have applied FITC-polysucrose 70 in mice[5,6]. In mice, a suprapubic urinary catheter is introduced into the bladder[6,20]. Urinary and plasma probes are subjected to gel filtration before the analysis of FITC-polysucrose 70 fluorescence. Similar as in rats, GFR was analyzed via radioactivity[6,20]. The second publication concerning the application of FITC-polysucrose 70 in mice uses a model of peritoneal permeability and, therefore, did not analyze mouse urine for FITC-polysucrose 70 fluorescence[22]. The presented method was, therefore, modified to facilitate urine sampling and analysis. Urine samples can be easily obtained through a noninvasive transurethral urinary catheter. Urine analysis for FITC-polysucrose 70 fluorescence is performed without any previous high-performance size exclusion chromatography or gel filtration. By referencing FITC-polysucrose 70 fluorescence to mouse urine creatinine, the measurement of GFR with a radioactive assay is not needed.

Increases in blood pressure enhance glomerular permeability. Therefore, blood pressure monitoring is essential whilst investigating glomerular permeability. The most accurate blood pressure measurements in mice are performed via a central arterial catheter[23] needing heparinization of the catheter to prevent clotting. We have experienced problems with hematuria after low-dose heparinization and urinary catheter placement. Therefore, we decided to perform the tail-cuff technique to measure blood pressure, which is noninvasive and does not need heparinization. Depending on the depth of anesthesia, blood pressure monitoring with the tail-cuff method is sometimes challenging. Blood pressure membranes need to be checked in advance and the position of the mouse should be optimized before blood pressure recording.

In addition to challenges in blood pressure measurement, this technique is also limited by producing arbitrary units of FITC-polysucrose 70. So far, this technique has only been investigated in animals and, therefore, its relevance to the human glomerular filter is still unknown. Time intervals to investigate increases in glomerular permeability depend on mouse urine production. Therefore, very short-term increases in glomerular permeability (in minutes) may be missed due to the lack of mouse urine production. In this protocol, FITC-polysucrose 70 is normalized to urine creatinine, which is removed from the blood mainly via glomerular filtration, but also by proximal tubular secretion. This will introduce an error when estimating glomerular permeability using this method, reducing the measured fractional clearance of polysucrose[24].

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DISCLOSURES:

The authors have nothing to disclose.

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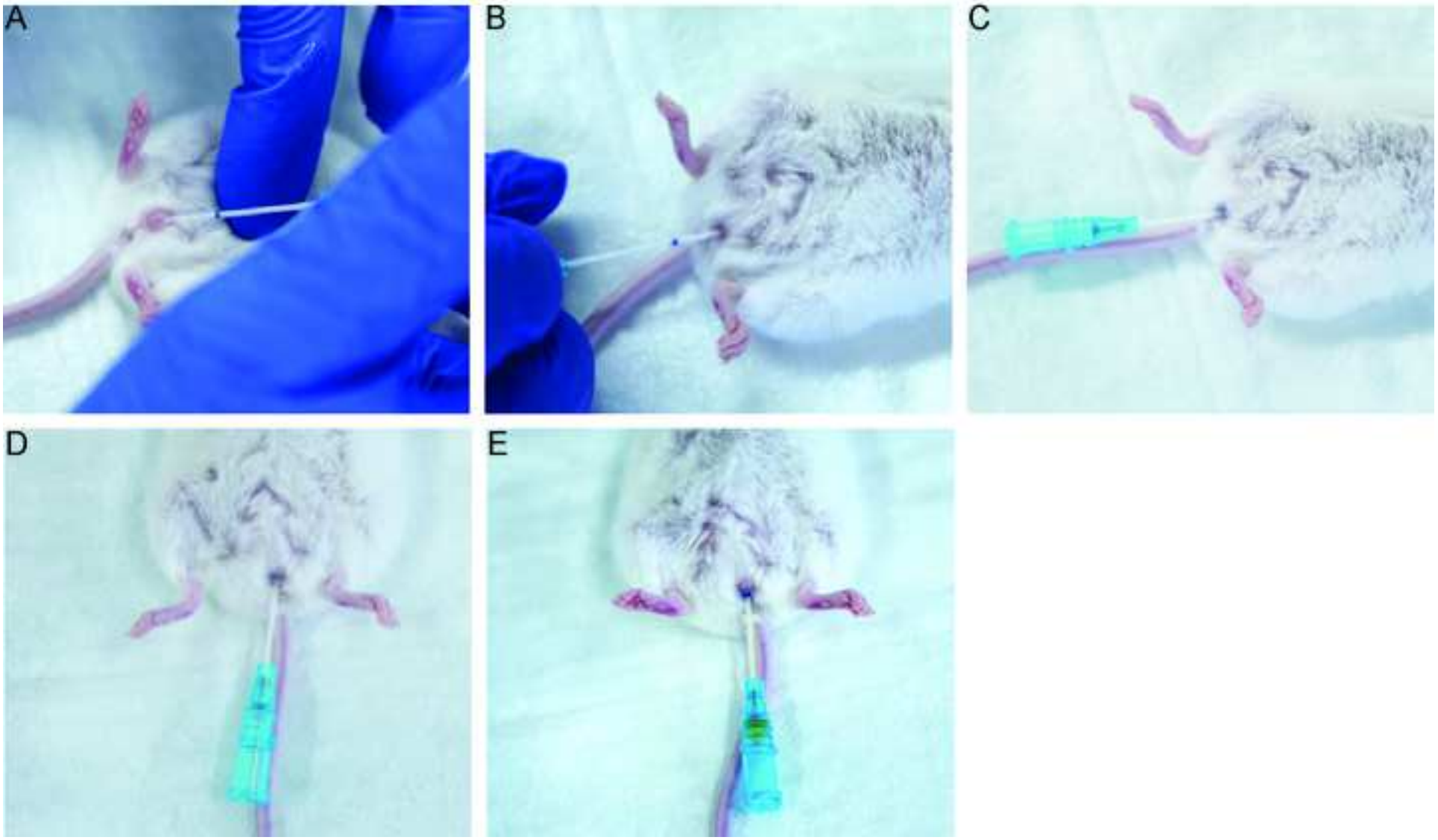

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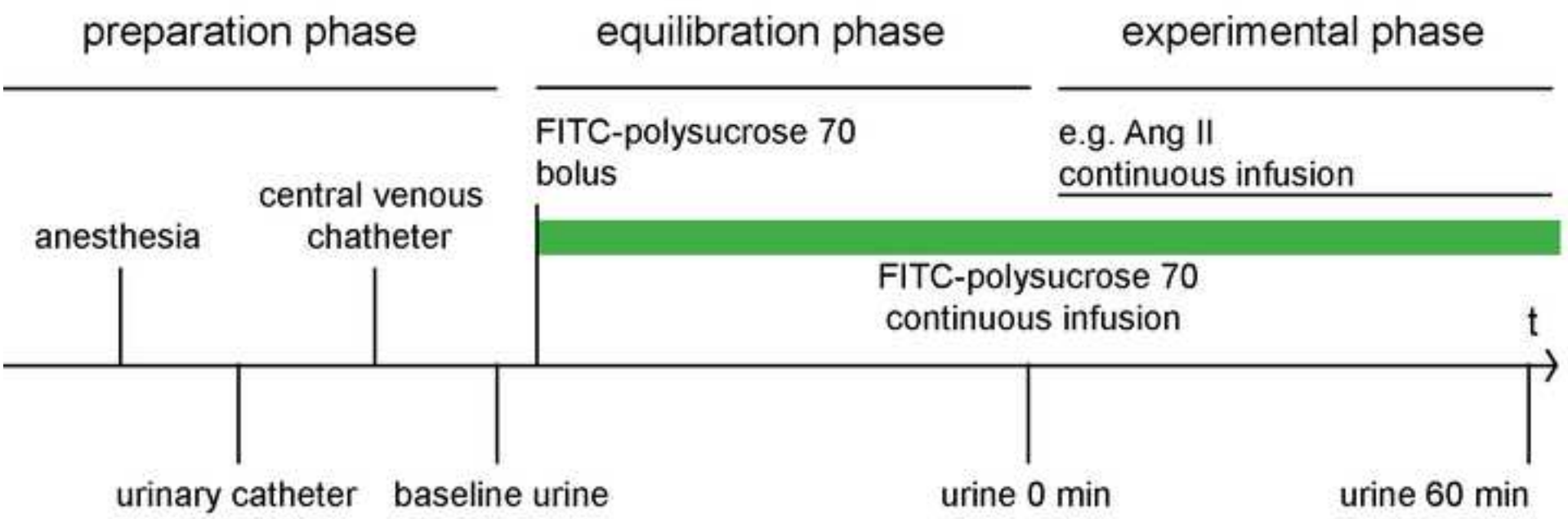

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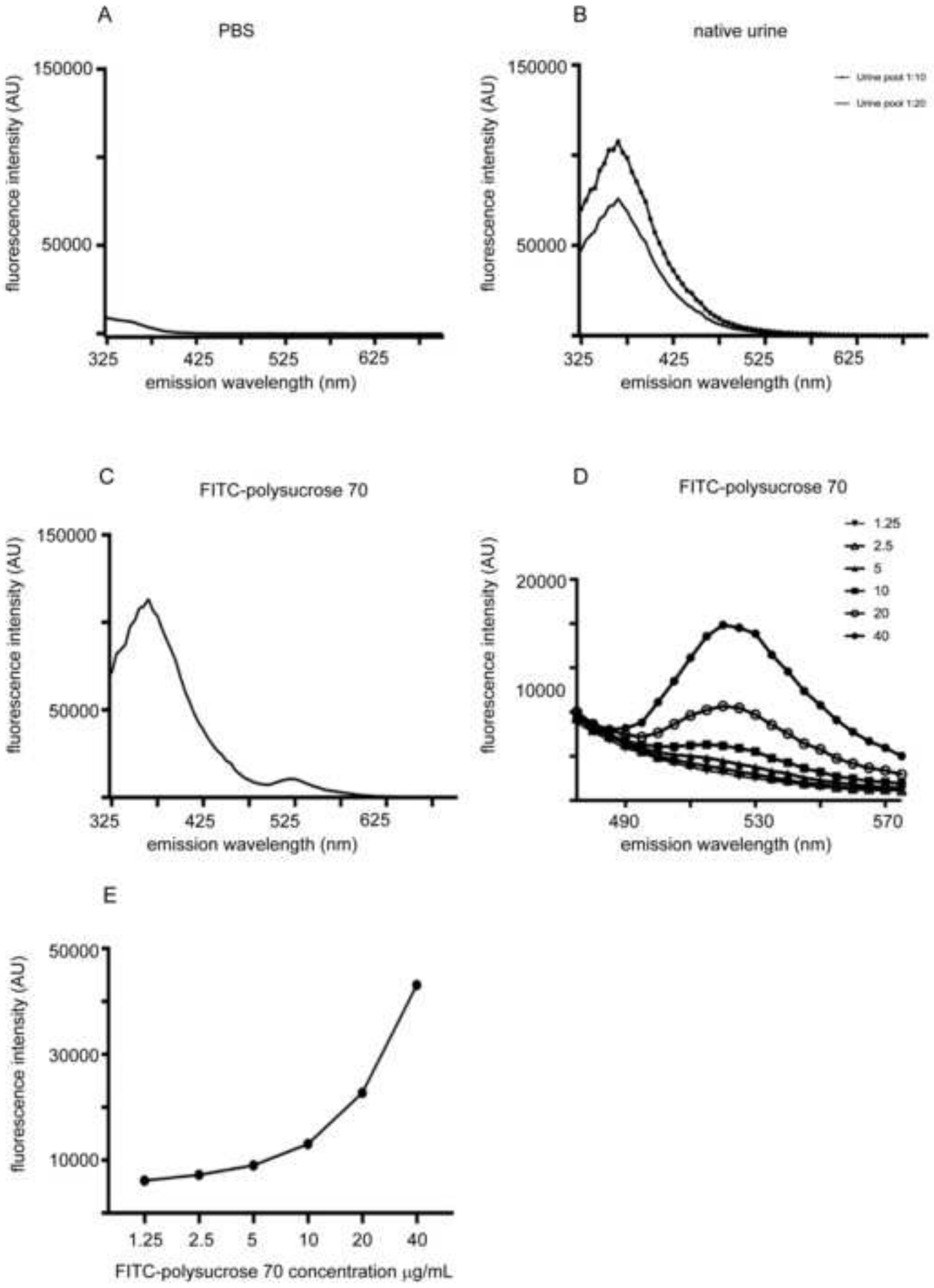

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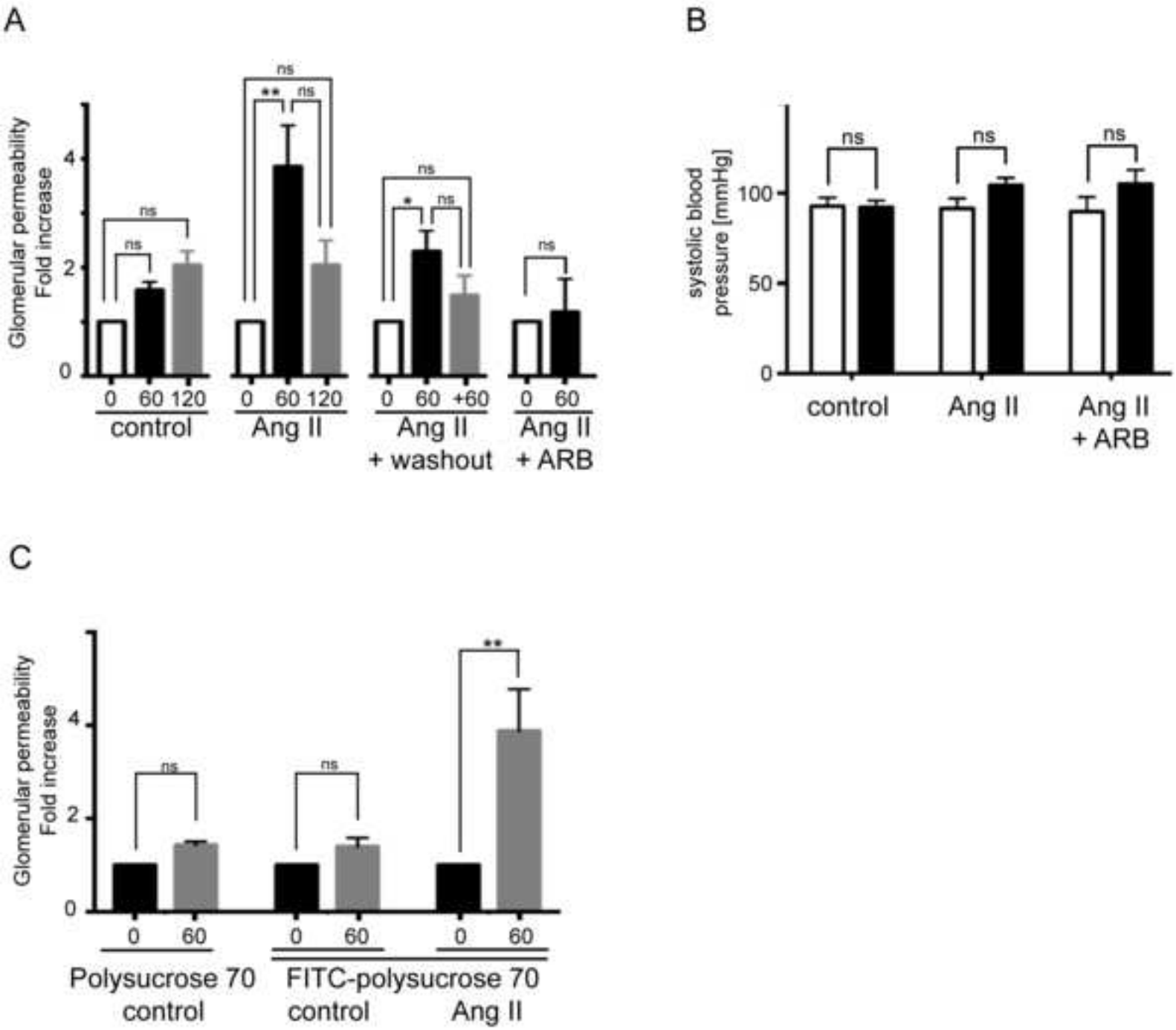

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KL1500LCD	Pulch and Lorenz microscopy	150500	light for mouse surgery
Microfederschere	Braun, Aesculap	FD100R	fine scissors
Durotip Feine Scheren	Braun, Aesculap	BC210R	for neck cut
Anatomische Pinzette	Braun, Aesculap	BD215R	for surgery
Präparierklemme	Aesculap	BJ008R	for surgery
Seraflex	Serag Wiessner	IC108000	silk thread
Ketamine 10%	Medistar		anesthesia
Rompun (Xylazin) 2%	Bayer		anesthesia
Fine Bore Polythene Tubing ID 0.28mm OD 0.61mm	Portex	800/100/100	Catheter
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BD Insyte Autoguard	BD	381823	urinary catheter
Multimode Detector DTX 880	Beckman Coulter		plate reader
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Creatinine Assay Kit	Sigma-Aldrich	MAK080	to measure creatinine concentration
96 well plate	Nunc	260836	for creatinine assay

FITC-labeled polysuccrose 70	TBD Consultancy	FP70	FITC-ficoll
Angiotensin II	Sigma-Aldrich	A9525	used to test glomerular permeability
BP-98A	Softron		for blood pressure measurement
OTS 40.3040	Medite	01-4005-00	heating plate for mouse surgery
Instillagel 6mL	Farco-Pharma GmbH		for urinary catheter
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CORRESPONDING AUTHOR:

Name: Lorenz Sellin

Department: Department of Nephrology

Institution: Heinrich-Heine-University

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Direktor: Univ.-Prof. Dr. L.C. Rump
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Klinik für Nephrologie

Direktor der Klinik
Univ.-Prof. Dr. L. C. Rump

European Hypertension Excellence Centre

Sekretariat
Frau Janssen
Tel.: (0211) 81-17726
Fax: (0211) 81-17722
e-mail:
Christian.Rump@med.uni-duesseldorf.de
Liliane.janssen@med.uni-duesseldorf.de

Stellvertreterin des Klinikdirektors und leitende Oberärztin
Prof. K. Ivens (0211) 81-16146
Geschäftsführender Oberarzt
Prof. L. Sellin (0211) 81-17723
Oberärzte:
Dr. A. Hansen (0211) 81-08136
Dr. E. Königshausen (0211) 81-16720
Dr. S. Kücükköylü (0211) 81-18928
Dr. S. Potthoff (0211) 81 18928
PD Dr. J. Stegbauer (0211) 81-17502
Dr. A. Wons (0211) 81-04163
Dr. M. Woznowski (0211) 81-04861
Oberarztsekretariat (0211) 81-17773

Stationen
ZA03 (Notaufnahme)
Tel.: (0211) 81-17012
Fax: (0211) 81-19518
MD 01 (Dialyseabteilung):
Tel.: (0211) 81-17763
Fax: (0211) 81-19797
Funk: 715 0048
ME04:
Tel.: (0211) 81-17843
Fax: (0211) 81-19798
ME05:
Tel.: (0211) 81-18927
Fax: (0211) 81-19519

Ambulanzen
Privatambulanz
Tel:. (0211) 81-17726
Sprechstunden: nach Vereinbarung

Nephrologische Ambulanz
Tel.: (0211) 81-18790
Fax: (0211) 81-18045
Sprechstunden: Mo-Fr. 8.00-16.00 Uhr
Transplantationsbüro
Tel.: (0211) 81-18627
Fax: (0211) 81-19282
Sprechstunden: nach Vereinbarung
KFH Nierenzentrum
Tel.: (0211) 81-18923 (Peritonealdialyse)
Fax: (0211) 81-19282
Tel.: (0211) 81-18923 (Hämodialyse)
Sprechstunden: Mo-Fr. 8.00-16.00 Uhr

Funktionsbereiche
Nephrologische Sonographie:
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Wissenschaftliches Labor:
Tel.: (0211) 81-10841, -18627

Studienzentrum:
Tel.: (0211) 81-17770

Webseiten der Klinik:
www.uniklinik-duesseldorf.de/nephrologie

UKD • Klinik für Nephrologie • Postfach 101007 • 40001 Düsseldorf

To
Ron Myers, Ph.D.
Science Editor
JoVE
1 Alewife Center, Suite 200
Cambridge, MA 02140
USA

Ansprechpartner:	Durchwahl:	E-Mail:	Datum:
Prof. Dr. L. Sellin	0211 81 17726	Lorenz.sellin@med.uni-duesseldorf.de	10.12.2018

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Dear Editor,

Thank you for the in-depth review of the manuscript. In our means your comments were very helpful to improve the manuscript substantially. Please find below the point by point reply:

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thank you very much for this comment. We have proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

1.1: Please list an approximate volume to prepare.

Response: We have added "*i.e.* 100 mg in 10 mL NaCl".

1.2: Please specify the dialysis tubing used (e.g., molecular weight cut-off).

Response: Thank you for your helpful comment. We added the molecular weight cut-off (10.000 Da) to point 1.2).

2.2.3, 2.2.4: Please specify surgical instruments used. How large is the incision?
Response: Thank you for this point. We have added that the skin incision is approximately 5 mm long. A pair of scissors and a tweezer are used.

2.2.9: Please provide the composition of infusion solution or refer to the previous preparation step.
Response: Thank you for this point. We have added preparation step 1.5.

4.7: Please describe how to sacrifice the mouse.
Response: Thank you for this helpful comment. We have added that the mouse is sacrificed by decapitation during anesthesia.

3. Please combine some of the shorter protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Response: Thank you for pointing this out. We have combined some shorter protocol steps.

4. Please include single-line spaces between all paragraphs, headings, steps, etc.
Response: Thank you for this point. We have included single-line spaces between all paragraphs, headings and steps.

5. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: We have highlighted the essential steps of the protocol for the video.

6. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.
Response: Thank you. We have highlighted as indicated.

7. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step

are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Response: Thank you for this helpful advice.

8. Please number the figures in the sequence in which you refer to them in the manuscript text.
Response: Thank you for pointing this out. We have numbered the figures in the sequence in which they are referred to in the manuscript.

9. Figure 4: Please define error bars in the figure legend.
Response: Thank you for pointing this out. We have defined the error bars in figure 4 within the figure legend.

10. Figure 4: As this figure was modified from a previous publication, it must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
Response: Thank you for this well taken point. We have added “This figure has been modified from [citation]” to figure 4.

Reviewer #1
Major concerns
1. The equilibration and experimental periods are much longer than that in in rats (ref 4). I guess that the longer times were chosen in order to achieve a sufficient amount of urine for analysis. However, this limits the method in that transient increases in glomerular permeability may not be accurately detected. This limitation should be mentioned in the Discussion.
Response: Thank you for this well taken point. We added this point to the discussion section. Shorter time intervals could be chosen depending on urine output, e.g. 30 minutes time points.

2. Creatinine is removed from the blood mainly via glomerular filtration, but also by proximal tubular secretion. This will introduce an error when estimating glomerular permeability using this method, reducing the measured fractional clearance of Ficoll. This limitation should be mentioned in the Discussion.
Response: Thank you very much for this helpful comment. We have added this comment to the discussion section.

Minor concerns

3. The authors could consider re-phrasing the first sentence in the introduction to be more general, e.g. to "Structural or functional alterations ...". Emerging evidence support the concept that cytoskeletal alterations within the cellular components (podocytes and ECs) of the glomerular filtration barrier (GFB) are key to explain the observed alterations in permeability occurring, for example those occurring during angiotensin II stimulus (ref 4). Such alterations may indeed be of a physiological or functional nature (e.g. proteinuria occurring after exercise) rather than being "defects" per se.

Response: Thank you for this helpful comment. We have added functional changes to the first sentence of the introduction.

4. Page 2 row 77: change "smallest" to "small".

Response: Thank you. We have changed smallest to small.

Reviewer#2

Major concerns:

1. This technique is completely dependent on the fluorescent readout of Ficoll-70 for relative changes. For figure 3, the authors state "the detector …is overdriven so that the diagram depicts a nick". I do not understand this at all. It appears to me that this "nick" is in the crucial part of the graph and that the important data is missing. Until this can be clarified, and a representative graph of the actual data is shown, this is unpublishable.

Response: Thank you for this well taken point. We have repeated the fluorescence scan of with urine pool probes (diluted 1:10 and 1:20 in PBS) and urine pool probes (1:10 in PBS) with increasing concentrations of FITC-Ficoll 70 (see figure 3 B+C). Figure 3B depicts the diluted urine pool fluorescence intensity depending on the wavelength. The peak auto fluorescence of the urine pool is at 365 nm. Figure 3C shows the peak fluorescence of urine at 365 nm and the FITC-Ficoll 70 fluorescence peak at 525 nm. Figure 3D shows only the FITC-Ficoll 70 fluorescence peak at 525 nm. Different concentrations of FITC-Ficoll 70 show increased fluorescence intensity measured with an emission at 525 nm (Figure 3E).

2. FITC as a conjugate is notoriously prone to detachment. The authors have attempted to remove free FITC dye, but have not demonstrated success. Also the amount of free dye as a consequence of being kept in the freezer over time was not considered or measured. These need to be addressed.

Response: Thank you for this well taken point. The manufacturer states concerning the stability of FITC-Ficoll (polysucrose) that the thiocarbamoyl linkage of fluorescein moiety and

Polysucrose is similar of the linkage to dextran. “The stability of FITC-dextran is in vivo and in vitro. Only at elevate pH (> 9) and elevated temperatures is there a risk for hydrolysis of the fluorescein label. Studies at 37 °C in rabbit plasma, muscle homogenate, liver homogenate and urine established that FITC-dextrans are stable for at least 3 days. No changes in the mol.wt and no release of fluorescein moieties was noted (Data sheet FITC dextran and FITC-polysucrose, TdB consultancy).” To address your questions we dialyzed FITC-Ficoll 70 and measured FITC-fluorescence in the dialysate. In addition, we performed a freeze and thaw cycle FITC-Ficoll 70 and dialyzed again to address the question whether the freeze and thaw cycle would affect hydrolysis of the fluorescence linkage to Ficoll. Fluorescence was again measured in the dialysate. We could only detect less than 1 ‰ of the FITC-Ficoll 70 probe in the dialysate.

3. The purpose of this adapted assay is to reduce technically complex alternative procedures. Authors very briefly describe the Rippe et al. method and list its disadvantages, ie. technically complex, radioactive, and invasive. However, because the authors do not discuss in any detail what this method involves, it's difficult to see how their method is less technically complex and invasive, particularly since the authors method involves surgical techniques that require precision, as they discuss in the discussion (331-333).
Response: Thank you for your comment. We have added a paragraph discussing the published methods in more detail and pointing out the advantages of the presented method.

4. The authors have not attempted to compare the changes that they have measured to what they would expect, based on the assay by Rippe et al or elsewhere in the literature. This is critical. The title suggests that this assay is "highly sensitive" but there is no evidence of this.
Response: Thank you for this comment. We have performed urine analysis with the albuwell assay and found that albumin/creatinine ratios do not differ significantly between 0 and 60 minutes in control and Ang II treated animals (Figure 1A for reviewers). Figure 1B for reviewers shows the albumin/creatinine ratio normalized to 0 minutes. Figure 1C for reviewers shows FITC-Ficoll 70 fluorescence/creatinine ratios for control and Ang II treated animals. Ang II treated animals display a significant increase compared to controls (3.9 times increase in Ang II treated animals compared to 1.3 times increase in control animals). Compared to data published in literature we studied changes in glomerular permeability. However, the assay published by Rippe and Haraldsson investigated the sieving coefficient. Direct comparison is therefore not applicable. We followed your suggestions using the DiaComp recommendations and used albuwell (see point Minor concerns 1).

Minor concerns:

1. Creatinine measurements are not ideal (and therefore not robust). DiaComp guidelines should be followed.

Response: Thank you for your comment. Creatinine measurements are not ideal as there is up to 30% tubular secretion in female mice (Eisner et al., Major contribution of tubular secretion of creatinine clearance in mice, Kidney Int. 2010). We checked the DiaComp website aiming to find guidelines, however we only found a protocol to measure creatinine in the urine using the Jaffé reaction. As shown in figure 2A increasing FITC-Ficoll 70 concentrations in the urine increase creatinine concentrations significantly when Jaffé reaction is used. We therefore performed an enzymatic creatinine measurement that does not interfere with FITC-Ficoll 70 in the urine (Figure 2B). We also followed the DiaComp protocol to measure albuminuria in mice using the albuwell assay (see Reviewer 2 major concerns point 4).

2. The authors need to prove that the Ficoll does not interfere with the creatinine assay rather than just assume that it is "unlikely".

Response: Thank you for pointing this out. We have measured creatinine concentrations of urine pool probes (1:10) with different FITC-Ficoll 70 concentrations. FITC-Ficoll 70 concentrations do not interfere with the enzymatic creatinine assay kit (Figure 2B for reviewers). However, FITC-Ficoll 70 concentrations (80 and 160 µg/mL) increase creatinine concentrations significantly that have been measured with the Jaffé reaction (Figure 2A for reviewers).

3. In the introduction, authors suggest that "researchers use animal models in which albuminuria is induced by robust renal injury due to toxins, drugs and renal surgery. However, induction of this type of renal injury often goes along with extreme stress to the animals". Stress to the animals would not be alleviated by this approach; long term investigations would still need to be done (and I would suggest that the stress is moderate, not extreme). It does not follow that a sensitive assay would negate the need for these models, just the need to measure albuminuria. This sentence is not relevant.

Response: Thank you for your comment. We have deleted the sentence within the introduction section.

4. Check point 1.4: final concentration of FITC Ficoll 70 would be 50 µg/ml not 40 µg/ml.

Response: Thank you for this helpful comment. We have changed the volumes so that the final concentration is 40 µg/ml.

5. Define "urinary pool". Is this urine without FITC Ficoll? There are two time points. How long is the urine collected for at each of these time points? The detail is lacking and it makes it difficult to understand. How does 4µl of a 10mg/ml FITC-Ficoll stock solution (i. e 4 µg), added to 250 µl urine equate to 160 µg/ml final concentration FITC Ficoll? By my calculations this gives 16 µg/ml.

Response: Thank you for bringing this up. A urinary pool is urine from 12-hour urine collection of healthy mice that is pooled. The urine is collected for 60 minutes after bolus injection (urine 60 min, equilibration phase). The urine is collected for further 60 minutes during the experimental phase (urine 120 min).

Concerning the concentration for the FITC-Ficoll 70 standards: 4 µl of a 10 mg/ml FITC-Ficoll 70 stock solution is equivalent to 40 µg FITC-Ficoll 70. If 40 µg of FITC-Ficoll 70 is added to 250 µl urine, the concentration is equal to 160 µg/mL.

6. Is 5.1.3 adding detail to 5.1.2? This is also unclear.

Response: Thank you for your comment. 5.1.2. describes how standard tubes are labeled. 5.1.3. describes how the standard 160 µg/mL is achieved. If this is confusing, we could combine both points.

7. The resolution of all of the figures is poor and needs improvement.

Response: Thank you for your helpful comment. We have assured high resolution of the figures.

8. Some errors in word usage and confusing sentences are present. They are listed below by line number:

48 Use of alternative is not necessary

Response: Thank you for your comment. We have changed the sentence accordingly.

52-55 These two sentences seem to be saying the same thing. If they are not, it's not clear how they are different.

Response: Thank for your helpful comment. We have deleted the word discrete within the first sentence. This hopefully helps to clarify our point. Albuminuria is a marker for cardiovascular outcome and glomerular injury. But albuminuria <30 mg/gC, i.e. in the normal range, is also associated with cardiovascular risk.

75-77 The lack of commas makes it unclear what this sentence is saying. Perhaps a comma

after suprapubic catheter application would help.
Response: Thank you for pointing this out. We have added a comma after suprapubic catheter application.

77 small instead of smallest.
Response: Thank you for your comment. We have changed smallest to small.

81 switch offers and therefore
Response: Thank you for this comment. We have switched offers and therefore.

106 one pair of fine scissors
Response: Thank you. We have changed it accordingly.

136 Be consistent. Use light protection tube here and later on say brown tube.
Response: Thank you for bringing this up. We have changed it to brown tube.

142 Not completely clear what it is to "over-expand the head of the of the mouse with a tape" is trying to say. I believe the authors are trying to say to hyper-extend the neck of the mouse with a piece of tape.
Response: Thank you for your helpful comment. We have changed it to hyper-extend the neck of the mouse with a piece of tape.

170 Unclear what leak tightness is. Authors can explain/reword and describe how you control for it.
Response: Thank you for your helpful comment. We reworded the sentence. “Control for tightness of the ligature by careful viewing through the microscope.”

213,215 Which collection timepoint in considered the urine pool. Need to clarify
Response: The urine pool is a pool of urine that has been collected from healthy mice in a 12-24 hour urine collection in a metabolic cage.

222 The use of respectively here may be incorrect.
Response: Thank you for your comment. We have deleted respectively.

215 Which collection timepoint in considered the urine pool. Need to clarify
Response: The urine pool is a pool of urine that has been collected from healthy mice in a

12-24 hour urine collection in a metabolic cage.

262 parallel to, not paralleling.
Response: Thank you for your comment. We have changed “paralleling” in line 268 into “parallel to”.

269 parallel to the murine spine, not paralleling
Response: Thank you for your helpful comment. We changed it as indicated.

292 use the singular, i.e. mouse. Not mice.
Response: We have changed mice into mouse.

305 "is aligning" should be, "is aligned to the murine spine".
Response: We have changed it as indicated.

313-314 This sentence is not clear and should also be two sentences.
Response: Thank you for this comment. As we have performed new fluorescence scans and changed the figure legends accordingly.

321 "FITC-ficoll 70 levels before". Before what?
Response: Thank you for your comment. FITC-Ficoll 70 levels before the begin of Ang II stimulation.

343 Lubrication should be used, not lubrification.
Response: Thank you. We have changed it to lubrication.

353 No comma needed after essential.
Response: The comma was removed.

372 Redundant saying "applied used".
Response: Thank you. Used was eliminated.

378 "enhance" should be "enhances"
Response: Thank you for this comment. We have changed “Increases in blood pressure enhance” to “Increase in blood pressure enhances”.

Reviewer #3

Major concerns:

1. The authors should use unlabeled ficoll as a control. Hence parallel mice treated without the fluorescent-label would provide a more accurate baseline for before and after permeability is induced, in this case by AngII. The unlabeled ficoll trace would then be subtracted from all the labeled experimental samples.

Response: Thank you for your helpful comment. We have performed additional mouse experiments with unlabeled Ficoll-70 as controls. Fluorescence/creatinine measurements do not show significant differences in mice treated with FITC-Ficoll. Please see figure 4C.

2. In the same context I would suggest to dilute the samples accordingly so that the peaks can be accurately measured.

Response: Thank you for this well taken point. We have repeated the fluorescence scan of with urine pool probes (diluted 1:10 and 1:20 in PBS) and urine pool probes (1:10 in PBS) with increasing concentrations of FITC-Ficoll 70 (see figure 3 B+C). Figure 3B depicts the diluted urine pool fluorescence intensity depending on the wavelength. The peak auto fluorescence of the urine pool is at 365 nm. Figure 3C shows the peak fluorescence of urine at 365 nm and the FITC-Ficoll 70 fluorescence peak at 525 nm. Figure 3D shows only the FITC-Ficoll 70 fluorescence peak at 525 nm. Different concentrations of FITC-Ficoll 70 show increased fluorescence intensity measured with an emission at 525 nm (Figure 3E).

3. Where is the urine peak (~395nm) in Figure 3C?

Response: The autofluorescence urine peak is found at 365 nm.

4. Authors should indicate whether mice were sacrificed at the end of the procedure and how this was done.

Response: Thank you for pointing this out. Mice were sacrificed by decapitation in anesthesia.

Minor concerns:

1. Figure 2. I find this figure not very informative. I suppose the video will address this, however a sketch diagram would be more effective here.

Response: Thank you for pointing this out. We have created a sketch diagram but feel that our art skills are not very elaborate. We therefore suggest to provide this figure within the supplemental material.

2. Figure 3. Use the same scale and increase font size.

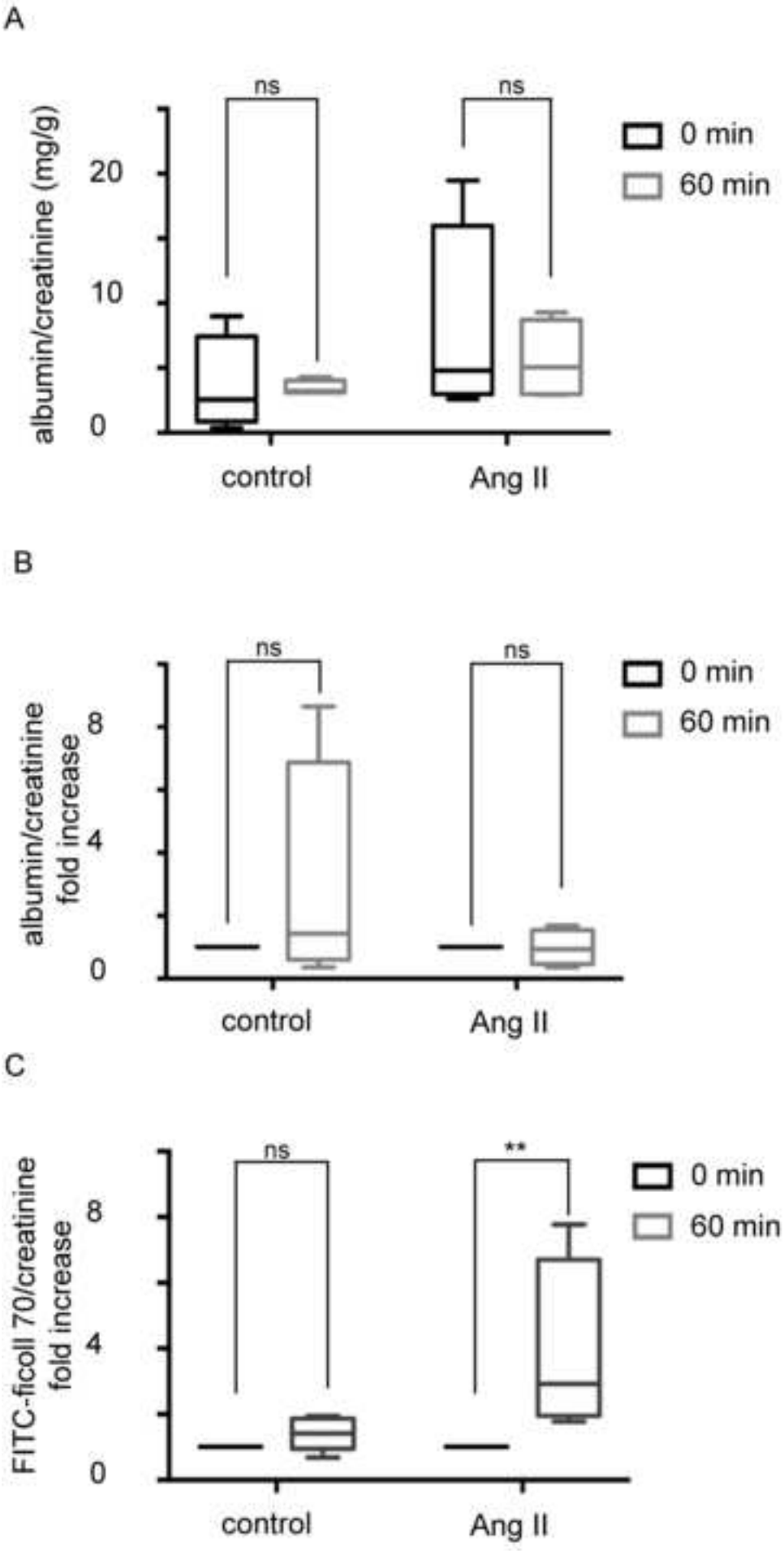
Response: Thank you for bringing this up. As we have performed new experiments, figure 3 was changed. We have used the same scale and increased the font size.

Yours Sincerely,

Lorenz Sellin, MD

Figure 1 for reviewers

Click here to access/download;Supplemental File (Figures, Permissions, etc.);figure 1 for reviewer.jpg



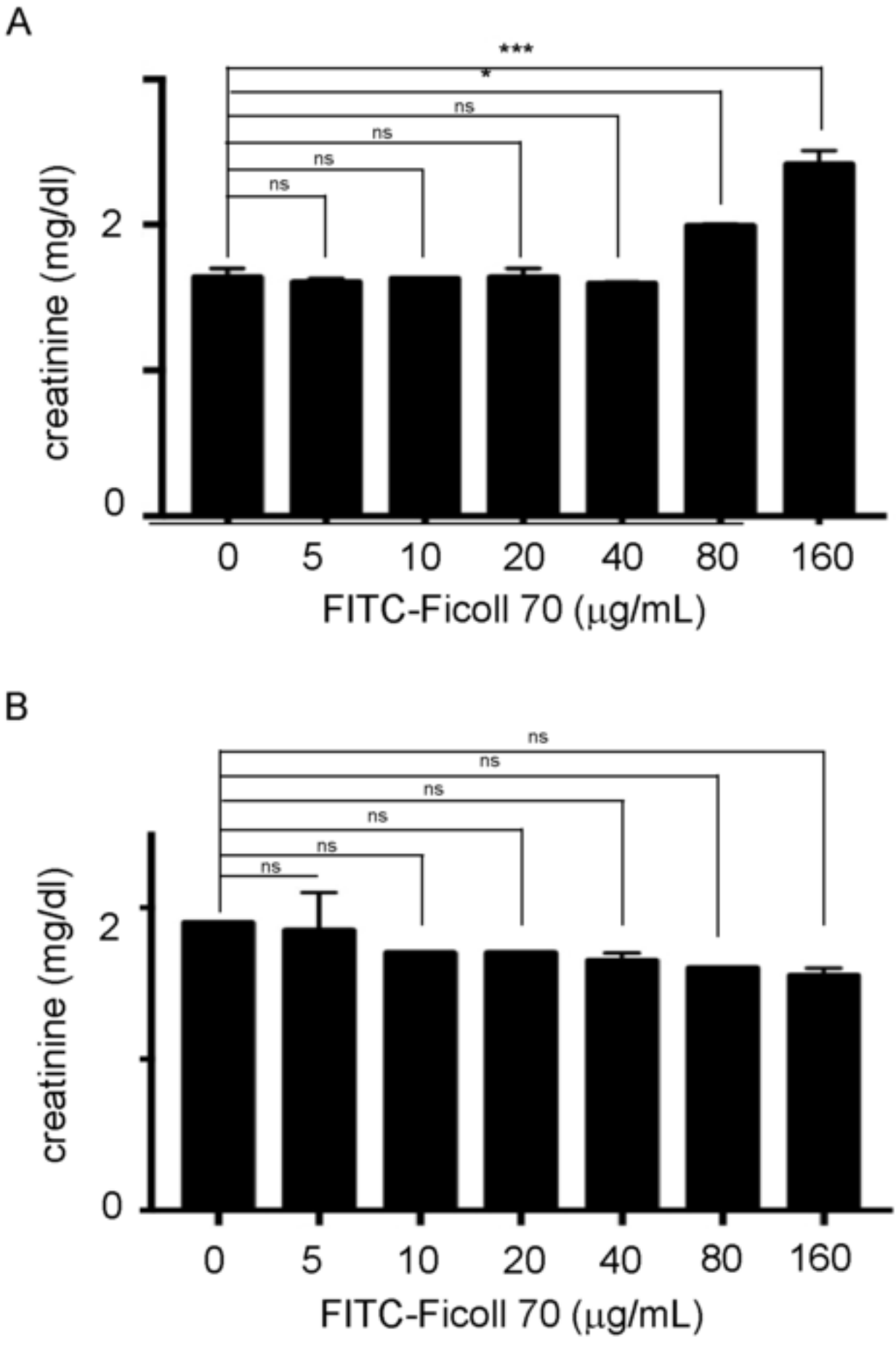


Figure 1 supplemental

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